Effects of Protease and Urea on a Granular Starch Hydrolyzing Process for Corn Ethanol Production

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ABSTRACT

The dry grind process using granular starch hydrolyzing enzymes (GSHE) saves energy. The amount of GSHE used is an important factor affecting dry grind process economics. Proteases can weaken protein matrix to aid starch release and may reduce GSHE doses. Two specific proteases, an exoprotease and an endoprotease, were evaluated in the dry grind process using GSHE (GSH process). The effect of protease and urea addition on GSH process was also evaluated. Addition of these proteases resulted in higher ethanol concentrations (mean increase of 0.3–1.8 v/v) and lower distillers' dried grains with solubles (DDGS) yields (mean decrease of 1.3–8.0% db) compared with the control (no protease addition). As protease levels and GSHE increased, ethanol concentrations increased and DDGS yields decreased. Protease addition reduced the required GSHE dose. Final mean ethanol concentrations without urea (15.2% v/v) were higher than with urea (15.0% v/v) in GSH process across all protease treatments.

A granular starch hydrolyzing enzyme (GSHE) converts granular starch into fermentable sugars at temperatures lower than starch gelatinization range (62–72°C for corn) (Robertson et al 2006). No cooking step is needed with GSHE compared with high-temperature hydrolysis with conventional enzymes. The dry grind process using GSHE (GSH process) was comparable (in ethanol concentration and fermentation rate) with a conventional dry grind process using traditional enzymes (α-amylase and glucoamylase) (Wang et al 2007). The GSH process can simplify the conventional dry grind corn process and reduce energy consumption by 10–20% (Robertson et al 2006). Granular starch hydrolysis is a solid-phase digestion process that differs from the soluble phase digestion process used in conventional dry grind. Solid starch hydrolysis requires a larger amount of enzyme compared with soluble starch hydrolysis (Kimura and Robyt 1995). GSHE is expensive compared with conventional enzymes; therefore, the amount of GSHE used is an important factor affecting dry grind process economics (Galvez 2005). Reducing the amount of GSHE in the GSH process is important to maintain the energy-saving benefit.

Starch granules in corn are encapsulated by endosperm-associated proteins in a protein matrix. Proteases degrade the protein matrix surrounding the starch granules and help release starch. Starch release by protease activity increased mash specific gravity and improved germ recovery in the enzymatic wet-milling process (Johnston and Singh 2001; Johnston et al 2003). During GSH processing, proteases may indirectly help starch hydrolysis by improving starch release from the protein matrix. In addition, proteases increase fermentation rates by hydrolyzing proteins into free amino nitrogen (FAN) (Lantero and Fish 1993). FAN produced by a protease (due to protein hydrolysis) could be substituted for an exogenous nitrogen source (urea, inorganic ammonium ions, or amino acids) needed by yeast during fermentation (Thomas and Ingledew 1990; Jones and Ingledew 1994). In ethanol production, urea is used as a nitrogen source for its high nitrogen content (46% N) and easy availability. Urea cost has increased 36% in the last three years (USDA 2007). Adding proteases could reduce GSHE usage and eliminate addition of other yeast nutrients.

Proteases are of two kinds: endoprotease and exoprotease. Endoprotease hydrolyzes peptide bonds anywhere along the protein chain. Exoprotease breaks peptide bonds from the amino or carboxy terminus and removes one amino acid from the protein chain at a time. In this study, two commercial acid proteases (exoprotease and endoprotease) used in the corn-processing industry were selected. Objectives of this study were to 1) investigate effects of endo- and exoproteases on the GSH process and 2) evaluate effects of protease addition on reducing or eliminating urea requirements in the GSH process.

MATERIALS AND METHODS

Experimental Material

Yellow dent corn (33D31 and 34M78; Pioneer Hi-Bred International, Johnston, IA) grown in 2005 and 2007 at the Agricultural and Biological Engineering Research Farm, University of Illinois at Urbana-Champaign, was used for the study. Samples were sieved using a 4.8-mm (12/64″) round-holed screen to remove broken corn and foreign material. Two proteases, an endoprotease (GC 106, *Aspergillus niger*; 1,000 spectrophotometer acid protease units/g; Genencor International, Palo Alto, CA) and an exoprotease (Novozym 50045, *A. oryzae*; 1,000 leucine amino peptidase units/g; Novozymes, Franklinton, NC), were obtained. GSHE (Stargen 001; *A. kawachi* and *A. niger*, 456 granular starch hydrolyzing units/g) was obtained from Genencor International. active dry yeast (Ethanol Red, Fermentis; Lesaffre Yeast Corporation, Milwaukee, WI) was used for the study. Yeast malt broth (ACS grade) was from Sigma-Aldrich (St. Louis, MO). Ammonium sulfite (ACS grade) and urea (99.6% ACS grade) were from Fisher Scientific (Fair Lawn, NJ).

Effect of Endo- and Exoproteases on the GSH Process

Cleaned corn samples (33D31; Pioneer Hi-Bred International, Johnston, IA) were ground in a 165-mm diameter hammer mill (model MHM4, Glen Mills, Clifton, NJ) at 500 rpm using a 2-mm sieve with round holes. The ground corn moisture content was measured by oven method (Approved Method 44-19) (AACC International 2000). Particle size analyses (Standard Method S319.3) (ASABE 2003) were performed in triplicate using a sieve shaker (model RX-86; W.S. Tyler, Cleveland, OH) equipped with

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U.S. standard sieves no. 20, 30, 40, and 50 (openings of 0.841, 0.595, 0.420, and 0.297 mm, respectively) and pan. Particle size distribution of ground flour was 40.6, 11.2, 13.2, and 5.2% on 20, 30, 40, and 50 screens, respectively, and 28.4% collected on the pan. Ground corn flour, 46.8%, passed through the 30 screen (595-μm diameter openings). In a 500-mL flask, 100 g of ground corn was mixed with water to obtain 32% dry solids content slurry. Using 10*N* sulfuric acid, mash was adjusted to pH 4.0 for GC 106 and pH 4.5 for Novozym 50045. Selection of pH was based on optimum pH for GSHE, proteases, and yeast. Optimum ranges of Stargen 001 and GC 106 are pH 4.0–4.5 and pH 2.5–3.5, respectively (Genencor International). Optimum range for Novozym 50045 is pH 4.5–6.0 (Novozymes).

The dry yeast (11 g of Ethanol Red) was rehydrated in 89 mL of distilled water containing yeast malt (1 g). Yeast cell suspension was maintained at 32°C for 20 min with 30 rpm agitation and had a cell count of 1.8×10^8 cells/mL using a Petrifilm plate (3M Company, St. Paul, MN). Granular starch hydrolysis and fermentation (GSHF) was conducted by adding 5 mL of yeast suspension, 0.25 g of $(NH_4)_2SO_4$ per 100 g of corn, GSHE, and proteases to ground corn slurry. GSHF was conducted in a flask placed in a shaking water bath (model SHKA 7000; Barnstead/Lab-line, Melrose Park, IL) with agitation (130 rpm) at 32°C for 72 hr. Agitation kept solids in suspension and improved mass transfer between enzymes and granular starch as well as between fermentable sugars and yeast. Agitation speed was selected based on results of a preliminary study. After 72 hr of fermentation, distillers' dried grains with solubles (DDGS) were recovered as total residue following fermentation (Wang et al 2005).

Fermentation was monitored by collecting 2-mL samples at 48 and 72 hr; samples were analyzed using HPLC (Waters, Milford, MA). Each sample was centrifuged at 13,362 × *g* for 3 min (model 5415D, Eppendorf, Germany). Preparation and analyses of HPLC samples were performed as described by Wang et al (2007).

Factorial experiments with three GSHE levels and four protease levels were conducted in triplicate. The manufacturer-recommended GSHE dosage was 1.0–2.5 kg/MT. GSHE levels of 1.1, 2.1, and 4.3 kg/MT (corresponding to 0.1, 0.2, and 0.4 mL/100 g of corn, respectively) were used. Based on protease activities and results from the preliminary study, endoprotease (GC 106) enzymes at 0, 0.1, 0.2, and 0.4 mL/100 g of corn and exoprotease (Novozym 50045) enzymes at 0, 0.2, 0.4, and 0.6 mL/100 g of corn were selected. Treatment without protease served as control. DDGS coproduct yield was calculated based on initial ground corn (db).

Comparison of Protease and Urea Addition on the GSH Process

Corn (34M78, Pioneer Hi-Bred International) grinding and ground corn particle size analyses were conducted as described previously. Particle size distributions of ground flour were 35.3, 19.8, 8.6, and 7.6% on 20, 30, 40, and 50 screens, respectively, and 27.4% on pan. Ground corn flour, 43.3%, passed through the 30 screen (595-μm diameter openings). In a 500-mL flask, 100 g of ground corn was mixed with water to obtain 30% dry solids content slurry. Slurry was adjusted to pH 4.0 using 10*N* sulfuric acid. The dry yeast (10 g of Ethanol red) was rehydrated in 50 mL of distilled water with agitation (30 rpm) at 32°C for 20 min. The yeast suspension had a cell count of 2.5×10^9 cells/mL using a Petrifilm plate (3M Company). GSHF was conducted by adding 1 mL of yeast suspension and GSHE with or without protease or 0.25 mL of 50% (w/w) urea (0.125 g/100 g of corn). Initial yeast cell count in the fermentation mash was 1×10^7 cells/mL. Samples (1 mL) were withdrawn from fermenters at 3, 6, 12, 24, 30, 48, 54, and 72 hr for HPLC analysis. After 72 hr of fermentation, DDGS was recovered as described by Wang et al (2005).

To investigate the influence of urea with protease addition, two sets of treatments with and without urea were conducted. Based on the protease study, GC 106 was selected as the protease for this experiment. For each set of treatments, four protease levels (GC 106 at 0, 0.05, 0.1, and 0.2 mL/100 g of corn) and three GSHE levels (0.1, 0.2, and 0.4 mL/100 g of corn) were selected. A factorial experiment design (4 × 3) was used. Each treatment was replicated three times.

Statistical Analysis

For the first experiment, ethanol concentrations at 48 and 72 hr and DDGS yields for different levels of exoprotease, endoprotease, and GSHE were compared using analysis of variance (ANOVA) for a two-factor treatment design (SAS Institute, Cary, NC). Similarly, for the second experiment, ethanol concentrations at 72 hr for different levels of endoprotease and GSHE, with and without urea, were compared using ANOVA for a two-factor treatment design. Individual treatment means for both experiments were compared using Duncan's multiple range tests. The level selected to show statistical significance was 5% ($P < 0.05$).

RESULTS AND DISCUSSION

Effect of Endoprotease and Exoproteases on the GSH Process

GSHE and protease levels significantly ($P < 0.001$) influenced ethanol concentration, although the interactions between these

TABLE I
Ethanol Concentrations at 48 and 72 hr with Varying Endoprotease, Exoprotease, and Granular Starch Hydrolyzing Enzymes (GSHE)[a]

	At 48 hr					At 72 hr				
	Ethanol concentration (% v/v) for endoprotease (mL/100 g of ground corn)									
GSHE dose	0	0.1	0.2	0.4	Mean (across endo)[b]	0	0.1	0.2	0.4	Mean (across endo)[b]
0.1	13.5	14.1	14.6	14.7	14.2a	15.1	16.3	16.3	16.7	16.1a
0.2	14.9	15.4	16.2	16.4	15.7b	15.7	16.9	17.4	17.8	17.0b
0.4	15.6	16.4	17.1	17.2	16.6c	16.2	17.6	17.8	18.0	17.4c
Mean (across GSHE)[b]	14.7A	15.3B	16.0C	16.1C	$LSD_{0.6}$[c]	15.7A	16.9B	17.2BC	17.5C	$LSD_{0.5}$[c]
	Ethanol concentration (% v/v) for exoprotease (mL/100 g of ground corn)									
GSHE dose	0	0.2	0.4	0.6	Mean (across exo)[b]	0	0.2	0.4	0.6	Mean (across exo)[b]
0.1	13.3	13.5	13.9	14.4	13.8a	15.2	15.2	15.6	15.7	15.4a
0.2	14.7	15.3	15.5	15.7	15.3b	16.0	16.3	16.6	16.6	16.4b
0.4	15.4	15.9	16.3	16.3	16.0c	16.4	16.7	17.0	17.2	16.8c
Mean (across GSHE)[b]	14.5A	14.9B	15.2C	15.4C	$LSD_{0.5}$[c]	15.8A	16.1B	16.4C	16.5C	$LSD_{0.4}$[c]

[a] Each value is the mean of three observations.
[b] Mean ethanol concentrations followed by the same letter in a column (abc) or row (ABC) within endoprotease or exoprotease treatment were not significantly different ($P < 0.05$).
[c] Least significant difference value for individual means in each protease at each fermentation time.

factors appeared to have no marked influence ($P = 0.65$). Ethanol concentrations increased with addition of endoprotease (Table I). Differences were observed in ethanol concentrations at 48 and 72 hr between endoprotease doses of 0.1 and 0.4 mL. No differences in ethanol concentrations at 72 hr were observed between endoprotease doses of 0.1 and 0.2 mL or between 0.2 and 0.4 mL. Compared with the control (no endoprotease addition) and across all GSHE treatments, addition of 0.1, 0.2, and 0.4 mL of endoprotease increased mean final (72 hr) ethanol concentrations from 15.7 to 16.9, 17.2, and 17.5% v/v, respectively.

Final (72 hr) ethanol concentrations of control treatments with 0.1, 0.2, and 0.4 mL of GSHE were 15.1, 15.7, and 16.2% v/v, respectively (Table I). However, final ethanol concentration of the treatment with 0.1 mL of GSHE and 0.1 mL of endoprotease was 16.3% v/v and higher than the final ethanol concentration of treatments with 0.2 mL of GSHE alone. Similarly, final ethanol concentration of the treatment with 0.2 mL of GSHE and 0.1 mL of endoprotease was higher than the final ethanol concentration of treatments with 0.4 mL of GSHE alone. These results indicated that endoprotease addition can reduce the GSHE dose in the GSH process.

Granular starch hydrolysis is a solid phase starch digestion process and requires fine grinding of corn (more surface area) to reduce the GSHE dose (Robertson et al 2006). Ethanol concentration at 72 hr for ground yellow dent corn (86% material passing through 0.59-mm screen openings) with 32% solid content slurry and 0.23 mL of GSHE (2.5 kg/MT) has been reported to be 17.7% v/v (Anonymous 2005). In this study, the ground corn with only 46.8% material passing through 0.59-mm screen openings and 32% solid content slurry with 0.20 mL of GSHE (2.1 kg/MT) resulted in a mean ethanol concentration (at 72 hr) of 15.7% v/v. However, addition of 0.1, 0.2, and 0.4 mL of endoprotease to the above corn slurry resulted in final ethanol concentrations (at 72 hr) of 16.9, 17.4, and 17.8% v/v, respectively. Final ethanol concentration achieved with 0.4 mL of endoprotease (17.8% v/v) was similar to the one reported by the enzyme company (17.7% v/v) (Anonymous 2005). These results indicated that endoprotease addition helps overcome the effect of large particle size distribution.

Ethanol concentrations increased with exoprotease addition (Table I). Differences were observed in ethanol concentrations between exoprotease doses of 0.2 and 0.4 mL and between 0.2 and 0.6 mL. No differences in ethanol concentrations were observed between exoprotease doses of 0.4 and 0.6 mL. Compared with the control (no exoprotease addition) and across all GSHE treatments, addition of 0.2, 0.4, and 0.6 mL of exoprotease increased mean final ethanol concentrations from 15.8 to 16.1, 16.4, and 16.5% v/v, respectively.

Ethanol concentrations at 48 and 72 hr increased with increase of GSHE dose (Table I). Mean ethanol concentrations at 48 and 72 hr across all endoprotease doses for 0.1 mL for GSHE were 14.2 and 16.1% v/v, respectively. As GSHE dose increased to 0.4 mL, mean ethanol concentrations at 48 and 72 hr increased to 16.6 and 17.4% v/v, respectively (Table I). Similarly, mean ethanol concentrations at 48 and 72 hr across all exoprotease doses increased with an increase in GSHE dose from 0.1 to 0.4 mL.

Glucose concentrations were 0–0.4% w/v for all treatments (GSHE, endoprotease, and exoprotease) at 48 and 72 hr (data not shown). Low glucose concentrations indicated that fermentations for all treatments were complete (with no stuck fermentation).

As the amount of endoprotease increased, DDGS yields decreased (Table II). Compared with the control treatment, DDGS

TABLE II
Distillers Dried Grains with Solubles (DDGS) Yields with Varying Endoprotease, Exoprotease, and Granular Starch Hydrolyzing Enzymes (GSHE)[a]

	DDGS yields (% db) for endoprotease (mL/100 g of ground corn)				
GSHE dose	**0**	**0.1**	**0.2**	**0.4**	**Mean (across endo)[b]**
0.1	46.1	42.4	40.9	39.7	42.3a
0.2	43.7	38.3	35.6	34.5	38.0b
0.4	41.4	35.9	34.0	32.9	36.0c
Mean (across GSHE)[b]	43.7A	38.8B	36.8C	35.7D	$LSD_{1.5}$[c]
	DDGS yields (% db) for exoprotease (mL/100 g of ground corn)				
GSHE dose	**0**	**0.2**	**0.4**	**0.6**	**Mean (across exo)[b]**
0.1	46.3	45.8	44.6	44.0	45.2a
0.2	42.5	41.0	40.2	40.4	41.0b
0.4	40.6	38.7	38.1	37.8	38.8c
Mean (across GSHE)[b]	43.1A	41.8B	41.0C	40.7C	$LSD_{1.2}$[c]

[a] Each value is the mean of three observations.
[b] Mean DDGS yields followed by the same letter in a column (abc) or row (ABC) within endoprotease or exoprotease were not significantly different ($P < 0.05$).
[c] Least significant difference value for individual means in each protease.

TABLE III
Ethanol Concentrations at 72 hr with Varying Urea, Protease, and Granular Starch Hydrolyzing Enzymes (GSHE)[a]

		Protease (mL/100 g of ground corn)					
Urea (g/100 g of ground corn)	**GSHE (mL/100 g of ground corn)**	**0**	**0.05**	**0.1**	**0.2**	**LSD[b]**	**Mean (urea)[c]**
0	0.1	13.3	13.7	14.3	14.5	0.7	15.2a
	0.2	14.8	15.4	16.2	16.2		
	0.4	15.4	15.8	16.5	16.7		
	Mean (protease)[c]	14.5A	15.0B	15.7C	15.8C		
0.125	0.1	12.9	13.3	13.6	13.8	0.7	15.0b
	0.2	15.2	15.3	15.8	15.9		
	0.4	15.7	15.8	16.0	16.4		
	Mean (protease)[c]	14.6A	14.8BC	15.1BC	15.4C		

[a] Each value is the mean of three observations.
[b] Least significant difference value for individual means with urea or without urea.
[c] Mean ethanol concentrations followed by the same letter in a column (abc) or row (ABC) were not significantly different ($P < 0.05$).

yields with the addition of 0.1 mL of endoprotease and 0.1, 0.2, and 0.4 mL of GSHE decreased 8, 12, and 13%, respectively. DDGS yields with 0.2 mL of endoprotease and 0.1 mL of GSHE was lower than with 0.2 mL of GSHE alone. Lower DDGS yields and higher final (72 hr) ethanol concentrations with endoprotease addition indicated that more fermentable substrate was fermented into ethanol rather than being lost in the DDGS fraction. Similar patterns (lower DDGS yields) with addition of exoprotease were observed. No differences were observed in DDGS yields between the 0.4- and 0.6-mL exoprotease dose.

DDGS yields decreased with increase of GSHE dose (Table II). Mean DDGS yield across all endoprotease doses for 0.1 mL of GSHE was 42.3%. However, with the 0.2- and 0.4-mL GSHE dose, DDGS yields decreased to 38.0 and 36.0%, respectively. Lower DDGS yields with increasing GSHE doses also were observed for exoprotease treatments.

Comparison of Protease and Urea Addition on the GSH Process

Protease addition increased final ethanol concentration (Table III). With no urea addition and 0, 0.05, and 0.1 mL of protease addition, mean ethanol concentrations increased from 14.5 to 15.0 and 15.7% v/v, respectively. There was an interaction between urea and protease. Treatment with urea (0.125 g/100 g of corn) in addition to protease resulted in ethanol concentrations similar to or lower than those with protease alone. Mean ethanol concentration with no urea addition and across all protease and GSHE treatments was 15.2% v/v. However, ethanol concentration with urea addition and across all protease and GSHE treatments was lower (15.0% v/v). At 0.1 mL of GSHE, final ethanol concentrations of the treatments with urea and the same amount of protease were higher than for the treatment with urea. Similar results were obtained at a dose of 0.2 and 0.4 mL of GSHE (data not shown). Sarath et al (2001) noted that urea reduced protease activity by denaturing the protease. Urea did not increase final ethanol concentration in the GSH process. These results are in contrast with the conventional dry grind process. Addition of urea in the conventional dry grind process increases ethanol concentration (Ingledew and Bellissumi 2007).

CONCLUSIONS

A specific exoprotease and endoprotease were selected to evaluate the effect of protease on GSH process. Addition of these specific proteases resulted in higher ethanol concentrations (mean increase of 0.3–1.8 v/v) and lower DDGS yields (mean decrease of 1.3–8.0% db) compared with the control (no protease addition). As level of proteases and GSHE increased, ethanol concentrations increased and DDGS yields decreased. Final mean ethanol concentrations without urea (15.2% v/v) were higher than with urea (15.0% v/v) in the GSH process. Fermentation with protease alone had a higher ethanol concentration than with urea alone or with both protease and urea.

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